

AMENDMENTS TO THE SPECIFICATION

Please amend the first paragraph on page 1 as follows:

This application is a continuation of U.S. Application No. 09/858,852, filed May 16, 2001, now abandoned. U.S. Application No. 09/858,852 This application claims the benefit of U.S. Provisional Application No. 60/204,509, filed May 16, 2000, which is incorporated herein in its entirety. The disclosures of these applications are hereby incorporated by reference in their entireties herein.

Please amend the paragraph beginning on page 14, line 26 as follows:

In other embodiments, the weak DNA-binding domain comprises two Cys₂His₂ (SEQ ID NO: 4) zinc fingers.

Please amend the paragraph beginning on page 18, line 35 as follows:

FIG. 3. Recognition helix sequences of fingers isolated by our selection. For candidates that were isolated multiple times (as judged by nucleotide sequence), the number of clones obtained is shown in parentheses. The consensus sequence(s) of fingers selected by phage display for each target subsite are also shown (ref 6, +denotes a positivey charged residue, denotes no discernible preference). Asterisks indicate candidates with a 2 bp deletion downstream of the sequence encoding the recognition helix. Arrows illustrate a few of the most plausible potential base contacts. (SEQ ID NOS 23 91, respectively, in order of appearance).

Please amend the paragraph beginning on page 19, line 23 as follows:

FIG. 9. This figure shows the results of a certain embodiment of the subject interaction trap assay wherein a DNA-sequence can be selected which interacts with a specific protein. (SEQ ID NOS 15 22, respectively, in order of appearance).

Please amend the paragraph beginning on page 19, line 26 as follows:

FIG. 10. This figure shows a certain embodiment of the subject ITS wherein a reporter gene under the control of a basal promoter is used to identify false positives arising from amplification of the reporter gene construct. (SEQ ID NO: 92).

Please amend the paragraph beginning on page 19, line 29 as follows:

FIG. 11. This figure shows a certain embodiment of the subject ITS wherein protein dimerization may be detected. (SEQ ID NO: 93).

Please amend the paragraph beginning on page 19, line 31 as follows:

FIG. 12. This figure shows another embodiment of the subject ITS wherein protein dimerization may be detected. (SEQ ID NO: 94).

Please amend the paragraph beginning on page 20, line 1 as follows:

FIG. 14. This figure shows the result of a certain embodiment of the ITS for isolation of a novel DNA binding domain from a library of random polypeptides wherein the polypeptide does not bind to the promoter region of either reporter gene. (SEQ ID NOS: 95 97, respectively, in order of appearance).

Please amend the paragraph beginning on page 20, line 4 as follows:

FIG. 15. This figure shows the result of a certain embodiment of the ITS for isolation of a novel DNA binding domain from a library of random polypeptides wherein the polypeptide non-specifically binds to the promoter region of both reporter genes. (SEQ ID NOS: 95 97, respectively, in order of appearance).

Please amend the paragraph beginning on page 20, line 7 as follows:

FIG. 16. This figure shows the result of a certain embodiment of the ITS for isolation of a novel DNA binding domain from a library of random polypeptides wherein the polypeptide specifically binds to the promoter region of one of the reporter genes. (SEQ ID NOS: 95 97, respectively, in order of appearance).

Please amend the paragraph beginning on page 20, line 10 as follows:

FIG. 17. This figure shows the alternative result the ITS embodiment shown in FIG. 16, wherein the polypeptide specifically binds to the promoter region of the other reporter gene. (SEQ ID NOS: 95 97, respectively, in order of appearance).

Please amend the paragraph beginning on page 32, line 31 as follows:

The term "zinc finger protein" or "ZFPs" or "zinc finger polypeptide" refers to proteins that bind to DNA, RNA and/or protein, in a sequence-specific manner, by virtue of a metal stabilized domain known as a zinc finger. See, for example, Miller *et al.* (1985) *EMBO J.* 4:1609 1614; Rhodes *et al.* (1993) *Sci. Amer.* Feb:56 65; and Klug (1999) *J. Mol. Biol.* 293:215 218. The most widely represented class of ZFPs, known as the C₂H₂ (SEQ ID NO: 4) ZFPs, comprises proteins that are composed of zinc fingers that contain two conserved cysteine residues and two conserved histidine residues. Over 10,000 C₂H₂ (SEQ ID NO: 4) zinc fingers have been identified in several thousand known or putative transcription factors. Each C₂H₂ (SEQ ID NO: 4) zinc finger domain comprises a conserved sequence of approximately 30 amino acids that contains the invariant cysteines and histidines in the following arrangement: -Cys-(X)₂-₄-Cys-(X)₁₂-His-(X)₃₋₅-His (SEQ ID NO: 1). In animal genomes, polynucleotide sequences encoding this conserved amino acid sequence motif are usually found as a series of tandem duplications, leading to the formation of multi-finger domains within a particular transcription factor. As used herein, "zinc finger protein" refers to known zinc finger proteins, or fragments thereof, or to novel polypeptides isolated by the methods of the invention.

Please amend the paragraph beginning on page 63, line 16 as follows:

A second class comprises proteins in which the DNA-binding domain is comprised of multiple reiterated modules that cooperate to achieve high-affinity binding of DNA. An example is the C₂H₂ (SEQ ID NO: 4) class of zinc-finger proteins, which typically contain a tandem array of from two or three to dozens of zinc-finger modules. Each module contains an alpha-helix capable of contacting a three to five base-pair stretch of DNA. Typically, at least three zinc-fingers are required for high-affinity DNA binding. Therefore, one or two zinc-fingers constitute

a low-affinity DNA-binding domain with suitable properties for use as a component in this invention. Examples of proteins of the C2H2 class include TFIIIA, Zif268, Gli, and SRE-ZBP. (These and other proteins and DNA sequences referred to herein are well known in the art. Their sources and sequences are known.)

Please amend the paragraph beginning on page 78, line 11 as follows:

In this report we describe the design and testing of an E coli-based selection method that can detect either protein-DNA or protein-protein interactions and that can handle libraries larger than 10^8 in size. We tested our new method by selecting C₂H₂ (SEQ ID NO: 4) zinc finger variants similar to those previously isolated by phage display (6, 13). The results of our selection, the rapidity of our method, and the versatility of the underlying transcriptional activation scheme suggest that this bacterial-based system should provide a useful tool for identifying and characterizing protein-DNA and protein-protein interactions.

Please amend the paragraph beginning on page 78, line 29 as follows:

The Gal11P-Zif123 fusion protein contains residues 263-352 of the yeast Gal11P protein (with a N342V mutation [14]) fused by a nine amino acid linker Ala-Ala-Ala-Pro-Arg-Val-Arg-Thr-Gly (SEQ ID NO: 5) to residues 327-421 of Zif268 (the region encoding the three zinc fingers). The phagemid pBR-GP-Z123 expresses the Gal11P-Zif123 hybrid protein from an IPTG-inducible lacUV5 promoter. The pBR-GP-Z12BbsI phagemid is analogous to pBR-GP-Z123 except that Zif finger 3 is replaced with a modified Zif finger 1 in which the sequence encoding residues -1 through 6 of the finger recognition helix is replaced by unrelated sequence (a "stuffer" fragment) flanked by BbsI restriction sites. All phagemids used in this study can be easily "rescued" from cells by infection with a filamentous helper phage; infectious phage particles produced by these cells contain single-stranded phagemid DNA.

Please amend the paragraph beginning on page 79, line 4 as follows:

The reporter construct that expresses HIS3 (P_{zif}-HIS3-aadA) has the Zif268 binding site sequence 5'-GCGTGGGCG-3' centered at base pair -63 relative to the transcription start site of a weak E. coli lac promoter derivative (the P_{wk} promoter). The three selection strain reporters change the zinc finger binding site of P_{zif}-HIS3-aadA, replacing the sequence

5'TCGACAAGCGTGGGCG3' (SEQ ID NO: 6) (bases -74 to -59 relative to the transcription start site) with sequences that should allow binding of the desired zinc finger variants:

5'CAAGGGTTCAGGGGCG3' (SEQ ID NO: 7) (for NRE), 5'GGCTATAAAAGGGGCG3' (SEQ ID NO: 8) (for TATA), or 5'TGGGACATGTTGGGCG3' (SEQ ID NO: 9) (for p53). Each of these reporters was transferred (by recombination) to an F⁺ episome encoding lacI^r repressor and then introduced into strain KJ1C in a single step essentially as previously described (15, J. K. J. & C. O. P., unpublished). The resulting strains were then each transformed with the pACL-.alpha.Gal4 plasmid to create the NRE, TATA, p53, and Zif "selection strains."

Please amend the paragraph beginning on page 82, line 9 as follows:

Zinc finger domains can bind DNA and activate transcription in *E. coli*. We

tested our new *E. coli*-based system by applying it to a problem previously studied using phage display: the selection, from a large randomized library, of zinc finger variants with altered DNA binding specificities (for review, see 21). However, before proceeding with selections, we first examined whether a wild-type zinc finger protein could bind DNA and activate transcription in our system. (Relatively little information was available on the activity of C₂H₂ (SEQ ID NO: 4) zinc finger proteins in bacteria.) To do this, we constructed fusion proteins containing fragments of the yeast Gal11P and Gal4 proteins that had previously been shown to interact with each other (10, 14). Thus, we fused a Gal11P fragment to the three zinc fingers of the murine Zif268 protein (creating the Gal11P-Zif123 protein), and we replaced the carboxy-terminal domain of the *E. coli* RNA polymerase α subunit with a Gal4 fragment (creating the chimeric .alpha.Gal4 protein). A Zif268 DNA binding site was positioned upstream of our P_{wk}-HIS3-aadA operon to create the P_{Zif}-HIS3-aadA operon (FIG. 1C), and this cassette was introduced into a *dhisB E. coli* strain in single copy to create the "Zif reporter strain."

Please amend the paragraph beginning on page 86, line 9 as follows:

For the NRE target subsite, an initial attempt using our new selection method yielded only one finger (NSGSWK) (SEQ ID NO: 10) that bound preferentially to the target sequence. Based on our existing knowledge of zinc finger-DNA recognition (reviewed in 21), one can postulate reasonable contacts between recognition helix residues of this finger and bases in the

primary strand of the NRE subsite (FIG. 3C). However, we were initially concerned by the relatively low frequency of fingers selected for this site, and we repeated the selection using an additional enrichment step in an attempt to isolate more fingers. The great majority of sequences isolated this way had the same amino acid sequence as the candidate originally selected (NSGSWK) (SEQ ID NO: 10) but two closely related sequences (NSGSHK (SEQ ID NO: 11)) and NHGSWK (SEQ ID NO: 12)) were also identified. These results suggested that we might have obtained a small number of clones merely because very few candidates in our library can pass the threshold set in our NRE selection.

Please amend the paragraph beginning on page 86, line 21 as follows:

As shown in FIG. 3C, the sequences of fingers isolated in our NRE selections do not match the consensus sequence for fingers selected by phage display. We performed several experiments to explore the basis of this difference: We first checked our library by sequencing random candidates to ensure that there was no drastic bias in nucleotide distribution and were able to rule this out as a plausible explanation (unpublished data). We then decided to directly introduce (in exactly the same context) one of the fingers (TRTNKS) (SEQ ID NO: 13) that had been selected by phage display (6) and see whether it could work in our system as a Gal11P-zinc finger fusion protein. We find that NRE selection strain cells expressing this TRTNKS (SEQ ID NO: 13) finger fusion protein grow very poorly on HIS selective medium whereas the same cells expressing the NSGSWK (SEQ ID NO: 10) finger fusion (obtained in our selections) grow robustly (unpublished data). The simplest explanation for this result is that the TRTNKS (SEQ ID NO: 13) finger fusion binds poorly to the NRE subsite and therefore only weakly stimulates HIS3 expression. This explanation is supported by our observation that earlier selections with the NRE subsite, using a prototype of our system in which zinc fingers were expressed from a much higher copy number phagemid, had yielded the TRTNKS (SEQ ID NO: 13) as well as the NSGSWK (SEQ ID NO: 10) finger (J.K.J. and C.O.P., unpublished data). This suggests that our current system sets a very stringent standard for the NRE selections and may account for why we isolated such a small number of specific candidates.

Please amend the paragraph beginning on page 87, line 1 as follows:

We also used our binding site preference assay to compare the specificity of the NSGSWK (SEQ ID NO: 10) finger we had selected for the NRE subsite with that of the TRTNKS (SEQ ID NO: 13) finger selected by phage display. In our bacterial-based assays, the NSGSWK (SEQ ID NO: 10) finger binds specifically to the NRE subsite and binds only very weakly to the TATA subsite. By contrast, the TRTNKS (SEQ ID NO: 13) finger binds only weakly to all four subsites (exhibiting a preference for the NRE and TATA subsites over the p53 and Zif subsites) (unpublished data). These results suggest that the NSGSWK (SEQ ID NO: 10) finger we selected actually binds more tightly and specifically in our system than the TRTNKS (SEQ ID NO: 13) finger identified earlier by phage display.

Please amend the paragraph beginning on page 87, line 9 as follows:

Each of our three selections also yielded a small percentage of fingers that bind non-specifically to all four DNA subsites tested. Surprisingly, all of these fingers match a consensus sequence of the form R+WL+L(SEQ ID NO: 14) (where + denotes a positively charged residue, FIG. 3D). These fingers are rich in positive charge and may make extra phosphate contacts. We also note that all of these fingers have a tryptophan residue at position 2 and thus would not have been present in the libraries used for earlier phage display experiments. This highly conserved set of non-specific fingers raises many interesting questions: What level of specificity is required for a zinc finger protein to function in our assay (and thus to what extent does the E. coli chromosome function as a non-specific competitor)? How do these fingers bind? Why is this particular class of non-specific fingers the only type selected in our system?

Please amend the paragraph beginning on page 92, line 15 as follows:

The ability to simultaneously and independently monitor the interaction of a single DNA-binding protein with multiple DNA binding sites within a single cell could be very useful for selecting proteins with differential activation at distinct DNA binding sites. Separate reporter constructs, each with a separate DNA binding site driving the expression of a reporter gene that encodes a fluorescent protein with unique fluorescent properties, is one way to achieve this goal. In order to create such a system using the bacterial two-hybrid ITS, we decided to use EGFP and

dsRed (RFP) as the two reporter genes since they have different fluorescent emission spectra, but can both be excited by the argon ion lasers (λ =488 nm) commonly found in FACS machines. The first reporter construct has a binding site for the Zif268 protein, a minimal pLac promoter and EGFP as the reporter gene. The second reporter construct has a binding site for the T11 protein (a protein selected as part of Example 1), uses a hybrid promoter consisting of the λ pRM promoter with its -35 region replaced with the -35 region of the pLac promoter, and has dsRed as the reporter gene. The sequence of the pLac promoter is:

CTTTACACTTTATGCTCCGGCTCGTATGTTGTGTCGA (SEQ ID NO: 2) and the sequence of the hybrid promoter is: CTTTACAATTTATCCCTTGGTC GGCTAGATTTACTCGAG (SEQ ID NO: 3).

Please amend the paragraph beginning on page 94, line 12 as follows:

In addition to selecting proteins that bind to a specific DBS, this bacterial ITS can also be used to select DBS's that interact with a specific protein. FIG. 9 shows the results for such an in vivo site selection to select DNA sequences that interact with the P53^{zf} protein. The consensus binding site, as determined by Wolfe et. al., JMB 285, p1917 1934 (1999), for the P53^{zf} protein is CXGGACACGTX (SEQ ID NO: 15) where X indicates no clear sequence preference at that position. A library of EGFP reporter plasmids containing the partially randomized binding site CGGGANNNNNG (SEQ ID NO: 16) was created (where N indicates a mixture of A, G, C, T) and introduced into host cells containing the α -Gal4 and Gal11p-P53^{zf} fusion proteins. These cells were then grown to saturation at 37°C. in LB media with the appropriate antibiotics and then 100 μ l of this culture was used to inoculate 10 ml of minimal media (as described in example 3) containing 10 ng/ml aTc and 100 μ M IPTG. These cultures were then incubated for 24 hours at 30.degree. C. on a rotating drum incubator. After incubation, one round of FACS sorting was performed on a Cytomation MoFlo multiple laser FACS sorter and individual EGFP positive clones were selected. Of 20 clones analyzed, 16 were EGFP positive (i.e. expressed at least 2 fold more EGFP than control cells). These 16 positive clones contained three unique P53^{zf} binding sites. The most abundant of these sites matched the consensus from the vitro site selection.